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Cyanobacterial Phycobilisomes

CHARACTERIZATION OF THE PHYCOBILISOMES OF *SYNECHOCOCCUS* SP. 6301*

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A procedure is described for the preparation of stable phycobilisomes from the unicellular cyanobacterium *Synechococcus* sp. 6301 (also known as *Anacystis nidulans*). Excitation of the phycocyanin in these particles at 580 nm leads to maximum fluorescence emission, from allophycocyanin and allophycocyanin B, at 673 nm. Electron microscopy shows that the phycobilisomes are clusters of rods. The rods are made up of stacks of discs which exhibit the dimensions of short stacks made up primarily of phycocyanin (Eiserling, F. A., and Glazer, A. N. (1974) *J. Ultrastruct. Res.* 47, 16-25). Loss of the clusters, by dissociation into rods under suitable conditions, is associated with loss of energy transfer as shown by a shift in fluorescence emission maximum to 652 nm. *Synechococcus* sp. 6301 phycobilisomes were shown to contain five nonpigmented polypeptides in addition to the colored subunits (which carry the covalently bound tetrapyrrole prosthetic groups) of the phycobiliproteins. Evidence is presented to demonstrate that these colorless polypeptides are genuine components of the phycobilisome. The nonpigmented polypeptides represent ~12% of the protein of the phycobilisomes; phycocyanin, ~75%, and allophycocyanin, ~12%.

Spectroscopic studies show that phycocyanin is in the hexamer form, $(\alpha\beta)_6$, in intact phycobilisomes, and that the circular dichroism and absorbance of this aggregate are little affected by incorporation into the phycobilisome structure.

The phycobiliproteins are a group of homologous light-harvesting proteins which carry covalently linked tetrapyrrole prosthetic groups (1). These conjugated proteins represent the major light-harvesting components of Photosystem II in cyanobacteria (blue-green algae) and red algae. There are four spectroscopically distinct major classes of phycobiliproteins: phycoerythrins (λ_{max}, ~565 nm), phycocyanins (λ_{max}, ~620 nm), allophycocyanin (λ_{max}, ~650 nm), and allophycocyanin B (λ_{max}, ~670 nm). *In vivo*, the phycobiliproteins are assembled into particles, phycobilisomes, attached to the outer surface of the photosynthetic lamellae (2). Phycobilisomes have been isolated both from cyanobacteria (3) and red algae (4, 5). Such preparations have been examined by electron microscopy, but the information on the details of their ultrastructure is limited (4-6).

Phycobilisomes were reported to consist solely of phycobiliproteins (3, 7). However, recently Tandeau de Marsac and Cohen-Bazire (8) demonstrated the presence of several colorless polypeptides in phycobilisomes. The validity of this observation has been challenged by Gantt (9) and by Mörschel *et al.* (5).

Spectroscopic studies on intact cells demonstrated that energy absorbed by phycoerythrin is transferred to chlorophyll *a* with an efficiency approaching 100% (10). In isolated phycobilisomes, the terminal energy acceptor, chlorophyll *a*, is not present, and energy is transferred to allophycocyanin B, *i.e.* excitation of phycoerythrin in these particles leads mainly to emission at ~675 nm, the fluorescence emission maximum of allophycocyanin B (11, 12). The major energy transfer pathway indicated by numerous spectroscopic studies is:

$$\text{phycoerythrin} \rightarrow \text{phycocyanin} \rightarrow \text{allophycocyanin} \rightarrow \text{allophycocyanin B}$$

Phycoerythrin is present in some but not all cyanobacteria and red algae, whereas the other three pigments are invariably present.

In this laboratory, we have chosen to characterize the phycobilisomes of the unicellular cyanobacterium *Synechococcus* sp. 6301 (*Anacystis nidulans*). This organism was selected for detailed study primarily because of its simple phycobiliprotein composition. *Synechococcus* sp. 6301 produces only three phycobiliproteins, C-phycocyanin, allophycocyanin, and allophycocyanin B. All three of these proteins have been purified to homogeneity and characterized with respect to subunit composition, prosthetic group content, aggregation behavior, and spectroscopic properties (13-16). Much of the sequence of the major phycobiliprotein, C-phycocyanin, from this organism is known (17, 18).

In this report, we present data on the ultrastructure, fluorescence properties, circular dichroism, and polypeptide chain composition of the phycobilisomes of *Synechococcus* sp. 6301.

EXPERIMENTAL PROCEDURES

Materials

Density gradient grade sucrose and Triton X-100 were obtained from Calbiochem. Acrylamide (electrophoresis purity), *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine were purchased from Bio-Rad, and 2-mercaptoethanol from Eastman. Sodium dodecyl sulfate (specially pure) was obtained from BDH Chemicals, Ltd. All other chemicals were obtained from commercial sources and were of reagent grade.

The molecular weights and sources of the polypeptide standards for SDS[1]-polyacrylamide gel electrophoresis were as follows: bovine pancreatic deoxyribonuclease I (31,000, Worthington), *Escherichia coli* alkaline phosphatase (43,000, Sigma), yeast phosphoglucose isom-

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; Na-K-PO_4, NaH_2PO_4 titrated with K_2HPO_4 to a given pH.

erase (51,000, Sigma), beef liver catalase (60,000, General Biochemicals), bovine serum albumin (66,000, Miles/Pentex), *E. coli* β-galactosidase (116,000, Boehringer). C-Phycocyanin (α subunit, 17,700; β subunit, 19,000) and allophycocyanin (α subunit, 17,600; β subunit, 18,200) from *Synechococcus* sp. 6301 were prepared as previously described (13).

Rabbit antiserum to *Synechococcus* sp. 6301 allophycocyanin was obtained as described previously (19). A partially purified γ-globulin fraction was obtained by fractional precipitation with ammonium sulfate (20) and was stored frozen in 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.5, at −20°C. Immunodiffusion tests showed that this antibody preparation gave strong precipitin lines against allophycocyanin and dissociated phycobilisomes, but did not cross-react with C-phycocyanin.

Synechococcus sp. 6301 was obtained from the Berkeley collection (21) and grown in 1-liter cultures of Medium BG-11 (21). Cells were grown at 30°C in warm white fluorescent light to a density of 0.5 to 1.0 g, wet weight/liter and used immediately after harvest.

Methods

Preparation of Phycobilisomes—Phycobilisomes were prepared by a procedure similar to that developed by Gray and Gantt (3) and modified by Tandeau de Marsac and Cohen-Bazire (8). All buffers contained 10^{-3} M 2-mercaptoethanol and 10^{-3} M sodium azide. All procedures were carried out at room temperature unless otherwise specified.

Cells were harvested by centrifugation, washed twice in 0.65 M NaH_2PO_4/K_2HPO_4 (Na-K-PO_4) buffer at pH 8.0, and resuspended in the same buffer at a concentration of 0.12 g, wet weight/ml. The suspension was broken by passage through a French pressure cell at 20,000 p.s.i., then incubated for 30 min in the presence of 1% (v/v) Triton X-100. Whole cells and membrane particles were subsequently removed by centrifugation at 31,000 × *g* for 30 min at 18°C. Centrifugation led to the formation of a green membrane/detergent layer at the top of an intensely blue layer. The blue supernatant was removed carefully, leaving behind the membrane/detergent layer and a trace amount of the blue supernatant on top of the pellet. The spectrum of this soluble fraction, greatly enriched for phycocyanin, is shown in Fig. 1. Aliquots (1.1 ml) were layered onto sucrose step gradients consisting of 1.0, 3.0, 3.0, 2.3, and 2.2 ml, of 2.0, 1.0, 0.75, 0.5, and 0.25 M solutions of sucrose, respectively, all in 0.75 M Na-K-PO_4, pH 8.0, then centrifuged in a Spinco SW 41 rotor at 24,000 rpm (98,000 × *g*) for 13 h at 18°C. The phycobilisomes were recovered as a deep blue band, free of detectable chlorophyll (Fig. 2), and were typically used within 6 h of recovery from sucrose density gradients for electron microscopy and spectroscopic studies. They were stable, however, for at least 2 weeks, showing little sign of dissociation when stored at 4°C as a concentrated solution directly from sucrose gradients.

Polyacrylamide Gel Electrophoresis—Electrophoresis on slab gels (0.8 mm thick) was performed with the discontinuous buffer system of Laemmli (22) and the apparatus of Studier (23). Gels were prepared by dilution of an aqueous stock solution containing 30% (w/v) acrylamide and 0.8% (w/v) *N,N'*-methylenebisacrylamide. The running gel of 12% acrylamide, 0.375 M Tris-Cl (pH 8.8), and 0.1% SDS and the stacking gel of 5% acrylamide, 0.125 M Tris-Cl (pH 6.8), and 0.1% SDS were polymerized with *N,N,N',N'*-tetramethylethylenediamine and ammonium persulfate. The electrode buffer contained 0.025 M Tris, 0.192 M glycine, and 0.1% SDS, pH 8.4.

Samples were prepared by diluting protein solutions to 0.5 to 1.0 mg/ml with an equal volume of a dissociation mixture containing 0.1 M Tris-Cl (pH 6.8), 2% SDS, 1 M 2-mercaptoethanol, 15% glycerol, and 0.004% bromphenol blue, then heated at 100°C for 1 min. Presence of high K^+ concentrations in the sample led to splitting of bands on the SDS gels. This phenomenon may be related to the very low solubility of the potassium salt of SDS. In the case of solutions containing high concentrations of potassium salts, such as phycobilisome samples, glacial acetic acid was added to 10% by volume. The acidified samples were dialyzed exhaustively against 0.1 N acetic acid, lyophilized, and then dissolved in the dissociation mixture at 1 mg of protein/ml. This procedure was necessary because some cleavage (proteolysis?) of high molecular weight phycobilisome polypeptides was observed when phycobilisomes were dialyzed against solutions of low ionic strength at neutral or alkaline pH.

Ten microliters (~10 μg of protein) of each sample were applied to the gel and electrophoresis performed at a constant current of 30 mA. The gels were then stained successively for 1 h each in (*a*) 0.003% Coomassie brilliant blue R-250, 25% 2-propanol, 10% acetic acid; (*b*) 0.003% Coomassie brilliant blue, 10% 2-propanol, 10% acetic acid; (*c*) 0.01% Coomassie brilliant blue, 10% acetic acid. Gels were destained with 10% acetic acid. Densitometer scans were performed in a RFT Scanning Densitometer, model 2950. For permanent record, the gels were rinsed with distilled water and dried on a Whatman No. 3MM filter paper backing in a Savant slab gel dryer. Bands containing less than 0.1 μg of protein were readily detected on gels treated in this manner.

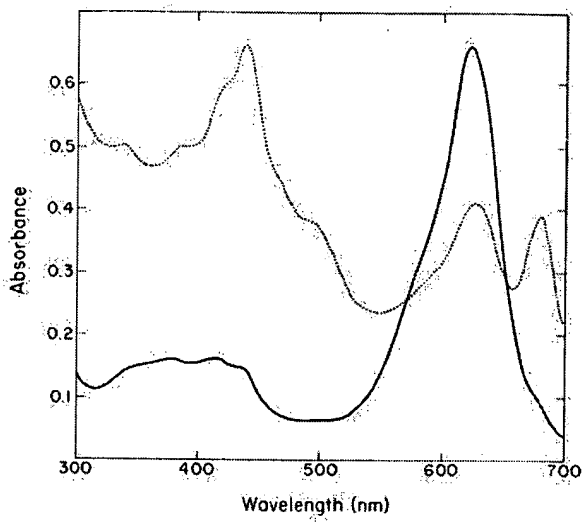

FIG. 1. Absorption spectra of broken cell suspension after a 100-fold dilution into 0.65 M Na-K-PO_4, pH 8.0 (· · · ·), and the soluble supernatant fraction after a 40-fold dilution into the same buffer (——). The latter fraction was obtained by incubation of the broken cell suspension in the presence of 1% Triton X-100 for 30 min followed by centrifugation for 30 min at 31,000 × *g*.

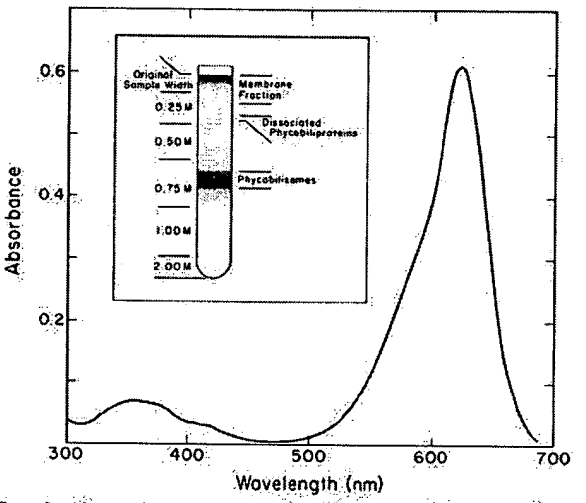

FIG. 2. Absorption spectrum of purified phycobilisomes, obtained as described under "Methods," in 0.75 M Na-K-PO_4, pH 8.0. *Inset* shows the profile of the sucrose step gradient after centrifugation of the soluble supernatant fraction described in the legend to Fig. 1. *Numbers on the left* represent the initial molar sucrose concentrations.

Spectroscopic Measurements—Absorption spectra were recorded at room temperature with Beckman model 25 recording spectrophotometer. Circular dichroism spectra were determined with a Cary model 60 recording spectropolarimeter at a sample absorbance of ~1/cm at λ_{max}. Fluorescence emission spectra were obtained on a Spex Fluorolog recording spectrofluorimeter at sample absorbance of 0.05 to 0.1 at λ_{max}. The emission spectra and maxima have been corrected for the variation with wavelength in the sensitivity of the detection system. The relative sensitivity of the instrument was 1.0 at 600 nm, 0.89 at 620 nm, 0.67 at 640 nm, 0.55 at 660 nm, 0.44 at 680 nm, 0.37 at 700 nm, 0.32 at 720 nm, and 0.31 at 740 nm.

Phycobilisome protein concentration was determined spectrophotometrically using the molar extinction coefficient of hexameric C-

phycocyanin of *Synechococcus* sp. 6301 $\epsilon_M^{621\ nm} = 3.33 \times 10^5\ M^{-1}\ cm^{-1}$ calculated for an $\alpha\beta$ molecular weight of 36,700 (15). C-Phycocyanin accounts for ~75% of the phycobilisome weight, while allophycocyanin represents ~12%. From the known ratio of 3:1 of the extinction coefficients of phycocyanin to allophycocyanin at 621 nm, the contribution of phycocyanin to the phycobilisome absorbance at 621 nm was estimated to be 95%. Phycobilisome concentrations were calculated from the C-phycocyanin concentration (in milligrams/ml) by dividing the latter value by 0.75.

Trypsin Digestion of Phycobilisomes—Two parameters were followed during tryptic degradation of phycobilisomes, proteolysis of phycobilisome polypeptides (as revealed by SDS-polyacrylamide gel electrophoresis) and the change in the fluorescence emission spectrum which accompanies dissociation of these particles. The reaction mixture (200 μl) contained 0.75 M Na-K-PO_4, pH 8.0, 0.75 M sucrose, 10^{-3} M 2-mercaptoethanol, and 600 μg of phycobilisomes. The reaction was started by the addition of 6 μl of trypsin solution (1 μg/μl) and allowed to proceed at room temperature. Samples were removed periodically in duplicate for analysis. Samples for gels were prepared by diluting a 15-μl reaction mixture aliquot with an equal volume of SDS-containing dissociation mixture (see above) and heating for 1 min at 100°C. Samples for fluorescence analysis were diluted 1 to 200 in 0.75 M Na-K-PO_4, pH 8.0, and emission spectra were determined within 2 min.

Electron Microscopy—Sample grids were of 400-mesh copper, coated with Formvar followed by deposition of a stabilizing film of carbon. They were made hydrophilic by treatment for 10 s to a glow discharge (~1000 V/cm) in a 70 to 80 millitorr partial vacuum. The phycobilisome samples, diluted in appropriate buffers to about 40 μg/ml, were applied to the specimen films in 5-μl drops and allowed to remain for 30 s. The grids were then rinsed in 100 mM ammonium acetate, followed by rinsing in 10 mM ammonium acetate, and staining in 0.5% aqueous uranyl acetate. Excess stain was removed by a Pasteur pipette flame-drawn to a fine bore. In some cases, the specimens were rotary-shadowed with tungsten (24), in which case, the step involving the uranyl acetate stain preceded the rinse in 10 mM ammonium acetate. Micrographs were obtained with a JEOL-100 B electron microscope and by use of the method of minimal beam exposure (25).

RESULTS

Phycobilisome Composition—Phycobilisomes were analyzed by gel electrophoresis in the presence of SDS and shown to contain five "colorless" polypeptides in addition to the colored tetrapyrrole-bearing subunits of the phycobiliproteins. Densitometer scans of gels stained with Coomassie brilliant blue indicated that phycocyanin accounted for about 75% of the protein, with allophycocyanin and the colorless proteins making up the remainder in nearly equal proportions (Fig. 3, and Table I). The bands originating from phycocyanin and allophycocyanin in the phycobilisome pattern were identified by comparison with those derived from purified phycocyanin and allophycocyanin. As illustrated in Fig. 3*A*, Bands 6, 7c, and 8 correspond to those from phycocyanin, while Bands 7a, 7b, and the leading edge of Band 8, correspond with those from allophycocyanin. Bands 6 and 8 are the β and α subunits of *Synechococcus* sp. 6301 phycocyanin, with molecular weights of 19,000 and 17,000, as determined previously by amino acid analysis (14) and sequence determination (18). Band 7c is a polypeptide also seen in purified phycocyanin, and it may perhaps be a subunit of a minor species of phycocyanin previously not detected on SDS-polyacrylamide gel systems of lower resolving power. Bands 8 and 7b represent the α and β subunits of allophycocyanin, reported earlier to have molecular weights of 16,200 and 17,400 (19). The present study indicates somewhat higher molecular weights of 17,600 and 18,200 for these subunits (see Fig. 3*B*). Band 7a, present in all phycobilisome gels analyzed, may be either a component of allophycocyanin or the β subunit of allophycocyanin B (16).

TABLE I

Quantitative analysis of Synechococcus sp. 6301 phycobilisome polypeptides

The Coomassie blue-stained Gel c of Fig. 3*A* was scanned at 600 nm in an RFT Scanning Densitometer with a slit width of 5.0 mm and a slit length of 0.2 mm in the absorbance mode. The scan profile is shown in Fig. 3*B*.

Band[a]	M_r	Protein content[b] (% by weight)	Identification of phycobiliprotein bands[c]
1	75,000	1.9	
2	45,000	0.4	
3	33,000	4.2	
4	30,000	1.9	
5	27,000	3.2	
6	19,000	38.2	Phycocyanin β subunit
7a	18,500	3.6	(Allophycocyanin β subunit)
7b	18,200	6.5	Allophycocyanin β subunit
7c	17,900	6.0	(Phycocyanin α subunit)
8	17,700	34.1	Phycocyanin α subunit and allophycocyanin α subunit

[a] Band numbers correspond to those shown in Fig. 3*B*.
[b] Numbers represent relative areas under the peaks in Fig. 3*B*.
[c] Identified by comparison with phycocyanin and allophycocyanin controls shown in Fig. 3*A*. Minor phycobiliprotein bands are given in parentheses.

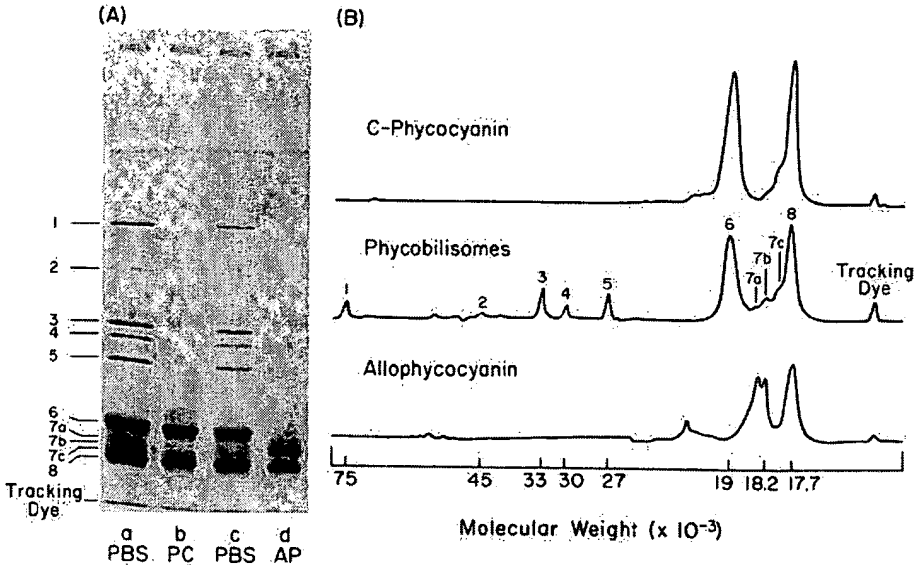

FIG. 3. *A*, polyacrylamide gel electrophoresis in the presence of SDS of (*a*) phycobilisomes (*PBS*), (*b*) phycocyanin (*PC*), (*c*) phycobilisomes, and (*d*) allophycocyanin (*AP*). *B*, densitometer scans of *Gels b*, *c*, and *d* in *Part A*. Molecular weights of the phycobilisome components are indicated by the numbers at the base of the scans.

The subunit molecular weights previously determined for the latter phycobiliprotein were 16,000 and 17,000 (16). This protein is present in a very small amount and its identification on gels is therefore difficult. However, fluorescence emission studies on our phycobilisome preparations demonstrate its presence.

The molecular weights of the colorless polypeptides were determined by SDS-polyacrylamide gel electrophoresis on gels calibrated with polypeptides of known molecular weight covering the range of 17,700 to 116,000. The data on the molecular weights and relative amounts of these components are presented in Fig. 3*B* and Table I.

Several lines of evidence support the view that the colorless polypeptides represent genuine structural components of phycobilisomes rather than adventitious contaminants.

When phycobilisomes isolated from the 0.75 M region of sucrose step gradients (described under "Methods") were diluted 4-fold into 0.75 M Na-K-PO_4, pH 8.0, and centrifuged through a 10 to 25% linear sucrose gradient in the same buffer, a single blue band was obtained containing each component of the original phycobilisome fraction in nearly unchanged proportions (see Fig. 4*B*, Gel c). The polypeptide composition

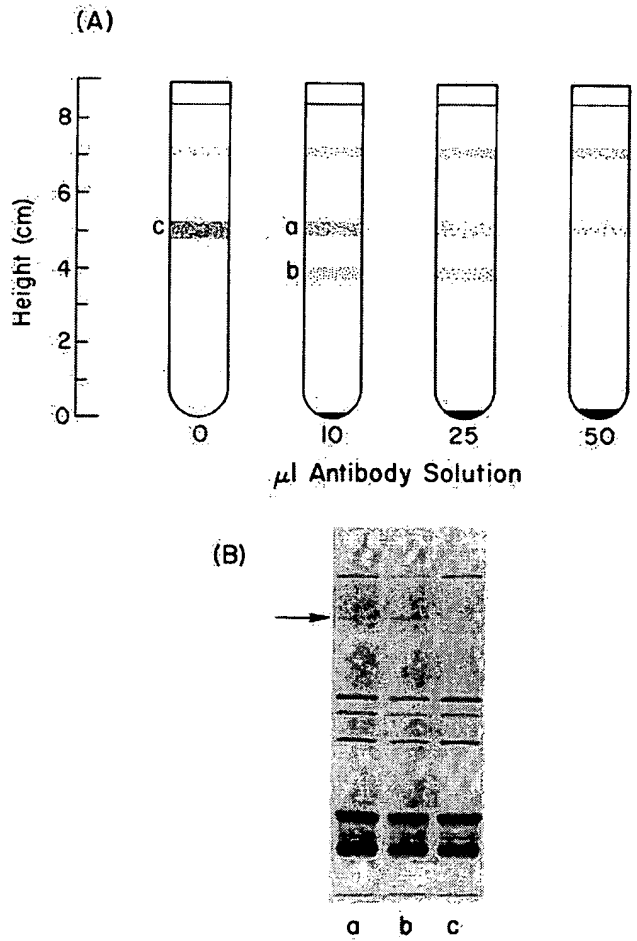

FIG. 4. *A*, linear sucrose density gradient profiles of phycobilisomes containing increasing amounts of anti-allophycocyanin antibody. Phycobilisomes were prepared as described under "Methods" and diluted 1 to 4 in 0.75 M Na-K-PO_4, pH 8.0, and 1-ml aliquots were mixed with the volumes of anti-allophycocyanin antibody solution indicated below each tube. Samples were applied to 10 to 25% linear sucrose density gradients in the same buffer, and after a 30-min incubation, were centrifuged in a SW 41 rotor at 24,000 rpm (98,000 × *g*) for 13 h at 18°C. *B*, SDS-polyacrylamide gel electrophoresis of *Bands a, b,* and *c*, identified in *Part A*. The *arrow* points to the band representing the heavy chain of the anti-allophycocyanin antibody.

of phycobilisomes isolated in a totally different buffer system (0.8 M sodium sulfate, 0.05 M Tris-Cl, pH 8.0) was the same as that of the particles isolated in 0.75 M Na-K-PO_4, pH 8.0.

The availability of highly specific anti-phycobiliprotein antibodies was exploited in another approach to this question. Antibody elicited against allophycocyanin was added to an excess of phycobilisomes to form a soluble antibody·phycobilisome complex separable from phycobilisomes on sedimentation in sucrose density gradients. In this experiment, different amounts of anti-allophycocyanin antibody were mixed with a phycobilisome sample and incubated for 30 min prior to centrifugation on a 10 to 25% linear sucrose density gradient. As shown in Fig. 4*A*, three bands appeared after centrifugation, consisting of (i) a slow sedimenting band of dissociated phycobilisome proteins, (ii) a phycobilisome band which sedimented at the same rate as untreated phycobilisomes, and (iii) a band of antibody·phycobilisome complex which sedimented significantly faster than the control phycobilisome band. The band of antibody·phycobilisome complex decreased in intensity as the amount of antibody solution added was increased from 10 to 50 μl/tube, concurrently with an increase in the amount of material in the pellet (Fig. 4*A*). The material in the band of antibody·phycobilisome complex (Fig. 4*A*, tube 2, Band b) was compared with that in Band a and in the control sample, tube 1, Band c (see Fig. 4*A*), by SDS-polyacrylamide gel electrophoresis. The sole significant difference between the antibody·phycobilisome complex and the control phycobilisome sample was the presence of a polypeptide of 50,000 daltons in the former sample, representing the heavy chain of γ-globulin. The light chain was not detected and may have co-electrophoresed with Band 5 (see Fig. 3). The significant observation was that all colorless polypeptides present in native phycobilisomes were also present in the antibody·phycobilisome complex (Fig. 4*B*).

In another experiment, phycobilisomes were prepared under conditions in which they were slowly dissociating (0.65 M Na-K-PO_4, pH 7.0). Sucrose density gradients of such preparations displayed a blue smear of material throughout the upper two-thirds of the gradient. When such gradients were fractionated throughout the pigmented region and the fractions were examined by SDS-polyacrylamide gel electrophoresis, the colorless components appeared in all pigmented fractions, and the relative intensity of each colorless component band relative to that of the other bands on the gel remained roughly constant.

Ultrastructure of Phycobilisomes—Preparations of purified phycobilisomes, examined after deposition upon the microscope grids in 0.75 M Na-K-PO_4 buffer at pH 8.0, invariably appeared as in Fig. 5*A* (negatively stained) and in Fig. 5*B* (rotary-shadowed). Just one type of structural element is seen, a disc 11.0 nm in diameter showing a midplane bifurcation when it appears edge-on. The discs are most frequently arranged in coaxial stacks to form rod-like assemblies. The rods, in turn, are frequently arrayed in clusters. The axes of the rods are rarely found in parallel aggregation, as would result from forces of surface tension during drying; indeed, they are seen at all angles to each other, including 90°C. We believe that Fig. 5*A* represents forms assumed by the phycobilisome material in the purified preparations; importantly, they also have the same general appearance as do the short stacks observed earlier in crude cell extracts of *Synechococcus* sp. 6301 (26).

The appearance of phycobilisomes, as seen in Fig. 5, *A* and *B*, shows more polydispersity than is suggested by the degree of compactness of the band formed during sedimentation through linear sucrose gradients. It is probable that some breakdown of the clusters occurs during preparation for elec-

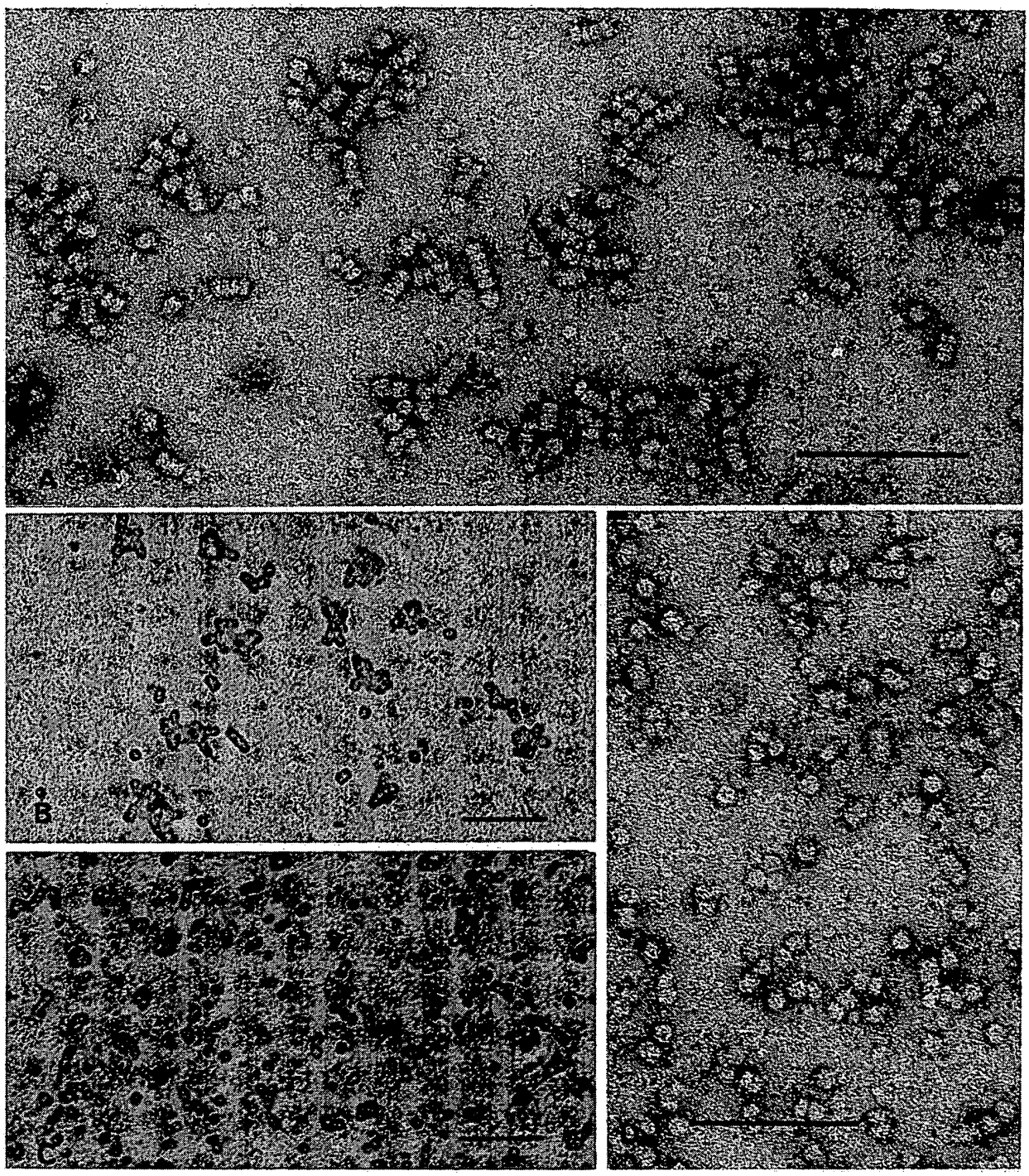

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FIG. 5. Electron micrographs of preparations of phycobilisomes. *A*, purified phycobilisomes diluted to 40 μg/ml in 0.75 M Na-K-PO_4, pH 8.0, negatively stained. Discs about 11.0 nm across are seen stacked to form short rods that are frequently clustered in random orientation. *B*, same sort of preparation as shown in *A*, but contrasted by rotary shadowing. *C*, starting material identical with that of *B*, but 15 min after dilution into 0.1 M sodium acetate, pH 5.5. Clusters have mostly disappeared, with single discs and rod-like stacks of discs remaining. *D*, phycobilisomes 6 h after dilution into 0.1 M sodium acetate, pH 5.5, negatively stained. Single discs predominate, along with some short stacks of discs. *Bars* drawn on micrographs represent 0.1 μm.

tron microscopy wherein the adsorbed phycobilisomes, just prior to negative staining, are rinsed in an ammonium acetate solution at only 0.01 M. In a trial experiment (not detailed here), we found that only 30 s after extensive dilution into 0.02 M Na-K-PO_4, pH 8.0, a phycobilisome preparation showed no clusters. Incorporation of glutaraldehyde, at 1% v/v, into the 0.75 M Na-K-PO_4, pH 8.0, used for the initial dilution had no effect on specimen appearance.

The dependence of energy transfer upon the existence of clusters was investigated. It had been found that the fluorescence emission properties of phycobilisomes are drastically affected by dilution into 0.1 M sodium acetate, pH 5.5, while the circular dichroism and absorption spectra are virtually unaffected (contrast Fig. 6 with Fig. 6, *insets A* and *B*). Phycobilisomes were diluted into 0.1 M sodium acetate, pH 5.5, to a final concentration of 40 μg/ml. After 15 min at room

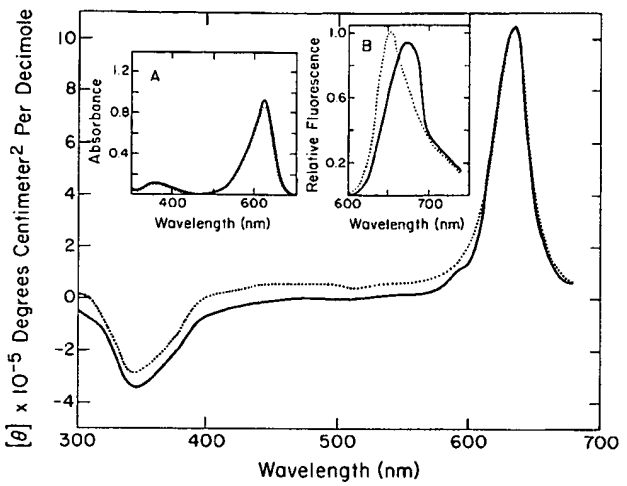

FIG. 6. Circular dichroism spectra of intact and partially dissociated phycobilisomes. Phycobilisome samples were diluted to an absorbance of ~1 at 620 nm either by dilution with 0.75 M Na-K-PO_4, pH 8.0 (——), or by passage through a column of Sephadex G-25 equilibrated and eluted with 0.1 M sodium acetate at pH 5.5 (····). The sample in acetate buffer was allowed to dissociate for 3½ h prior to determination of the circular dichroism spectrum. *Inset A*, absorption spectra of the samples used for the CD measurements, taken immediately after determination of the CD spectra. *Inset B*, corrected fluorescence emission spectra of solutions of phycobilisomes (14 μg of protein/ml) in 0.75 M Na-K-PO_4, pH 8.0 (——), and 0.1 M sodium acetate, pH 5.5, incubated at room temperature for 15 min prior to analysis (····). Excitation was at 580 nm, and excitation and emission slits were set at a 4 nm bandpass.

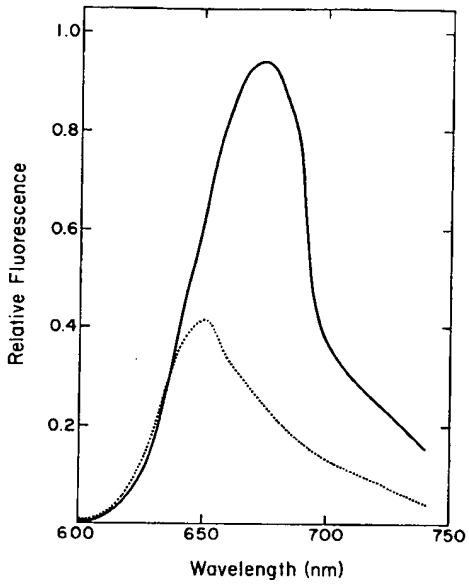

FIG. 7. Corrected fluorescence emission spectra of solutions of phycobilisomes (14 μg/ml) determined immediately after dilution into 0.75 M Na-K-PO_4, pH 8.0 (——), or dilution into distilled water (to give a buffer concentration of 0.004 M Na-K-PO_4) with 15 min of incubation at room temperature prior to analysis (····). Excitation was at 580 nm, and excitation and emission slits were set at a 3.2 nm bandpass.

temperature, the diluted solution was examined by fluorimetry and electron microscopy. The fluorescence emission maximum shifted from 673 to 652 nm, indicating virtual absence of energy transfer to allophycocyanin and allophycocyanin B (see Fig. 6, *inset B*). Electron microscopy of the sample showed that even this brief exposure to a buffer at low ionic strength and a pH of 5.5 caused dissociation of the clusters into rods and discs (compare Fig. 5, *B* and *C*). Although prolonged residence, 6 h, of the phycobilisomes in this buffer caused the complete dissociation of rods into discs (Fig. 5*D*), no further change occurred in the fluorescence emission spectra. Thus, we conclude that the preservation of the clusters is essential for retention of energy transfer through allophycocyanin and allophycocyanin B.

Spectroscopic Properties of Phycobilisomes—Phycobilisomes isolated from sucrose step gradients exhibited an absorption maximum at 624 nm (Fig. 2). The λ_{max} was independent of protein concentration over the range of 1.7 to 0.14 mg/ml in 0.75 M Na-K-PO_4, pH 8.0. When light of 580 nm, absorbed mainly by phycocyanin, was used to excite phycobilisomes, the fluorescence emission maximum was at 673 nm (Fig. 7). Comparison of the emission spectrum with those obtained from pure allophycocyanin and allophycocyanin B (11) showed that the emission peak was broader than that obtained from allophycocyanin B alone and slightly blue-shifted, indicating contributions from both allophycocyanin (fluorescence emission maximum at 660 nm) and allophycocyanin B. A similar observation was reported by Searle *et al.* (12) for the fluorescence emission spectrum of *Porphyridium cruentum* phycobilisomes.

Earlier studies of the pH dependence of the aggregation of *Synechococcus* sp. 6301 C-phycocyanin demonstrated that the protein existed primarily as the hexamer, $(\alpha\beta)_6$, at pH 5.0 to 6.0 and $\Gamma/2$ of ~0.1, whereas at pH 8.0, a monomer-trimer equilibrium predominated (13, 15, 27). Prolonged exposure (~6 h) of phycobilisomes, at a protein concentration of 0.14 mg/ml, to 0.1 M sodium acetate at pH 5.5 led to a near complete dissociation of these structures to small aggregates, most of them disc-shaped (Fig. 5*D*). The absorption spectrum was little perturbed by this treatment, a small shift in λ_{max} from 624 to 623 nm was observed (Fig. 6, *inset A*). The long wavelength positive CD band in the circular dichroism spectrum was unaltered (Fig. 6). This band, centered at 633 nm, was earlier shown to be characteristic of the hexameric aggregate of C-phycocyanin (15). Since the position and magnitude neither of this band nor of the absorption band are significantly altered upon dissociation of phycobilisomes at pH 5.5, it can be concluded that the spectroscopic properties of phycocyanin in phycobilisomes are very similar to those of phycocyanin in the $(\alpha\beta)_6$ assembly form. The spectroscopic properties of phycocyanin are, therefore, primarily the consequence of interactions between phycocyanin subunits within the hexamer (15) and are little affected by the interaction of phycocyanin with other proteins within the phycobilisome.

When phycobilisomes were dissociated in 0.06 M Na-K-PO_4, pH 8.0, the amplitude of the positive Cotton effect in the CD spectrum decreased by about 30% and a shoulder appeared on the short wavelength side of the peak. These changes are diagnostic of the trimer and monomer forms of C-phycocyanin (15). The fluorescence emission maximum under these conditions shifts to 650 nm, and the relative fluorescence is much lower than that of undissociated phycobilisomes (Fig. 7, compare with Fig. 6, *inset B*). These changes are consistent with the dissociation of phycocyanin into trimers and monomers, which have significantly lower extinction coefficients than the hexamer (15).

Partial Tryptic Digestion of Phycobilisomes—A short exposure (15 min at room temperature) of phycobilisomes in 0.75 M Na-K-PO_4, pH 8.0, to trypsin at an enzyme/substrate ratio of 1:100 by weight resulted in the complete disappearance of polypeptides 1 and 2 on SDS-polyacrylamide gels and a small shift in the fluorescence emission maximum from 673 to 669 nm. Absorption and circular dichroism spectra were unchanged. It is not known whether the cleaved polypeptides 1 and 2 are still bound to phycobilisomes or not. Electron

microscopy indicated that the short trypsin treatment did not lead to dissociation of the clusters.

DISCUSSION

Gantt and Lipschultz (4) have developed a procedure for the purification of phycobilisomes from the unicellular red alga *P. cruentum*. Phycobilisomes from this organism are prepared in 0.75 M Sorensen's phosphate buffer at pH 7.0 containing 1% Triton X-100. Under these conditions, the bulk of the phycobiliproteins migrate as a single rapidly sedimenting band on sucrose step gradients (4). Phycobilisomes prepared in this manner were reported to exhibit a fluorescence emission maximum at 672 nm when excited at 560 nm (12). When the above procedure was applied to the isolation of phycobilisomes from *Synechococus* sp. 6301, stable phycobilisomes were not obtained. Sucrose density gradients showed a marked heterogeneity in the size of the pigmented aggregates, and the fluorescence emission maximum of the largest aggregates lay at ~650 nm.

The following approach was adopted to the development of a rational procedure for the preparation of *Synechococcus* sp. phycobilisomes. The unicellular red alga *Porphyridium aerugineum* possesses the same complement of phycobiliproteins as *Synechococcus* sp. 6301, *i.e.* C-phycocyanin, allophycocyanin, and allophycocyanin B. In preliminary experiments, we found that in contrast to the results obtained with *Synechococcus* sp. 6301, relatively stable phycobilisomes from *P. aerugineum* could be obtained by the procedure for *P. cruentum* phycobilisomes cited above. The stability of the *P. aerugineum* phycobilisomes was then studied as a function of ionic strength, type of salt used, and pH. These exploratory experiments demonstrated that *P. aerugineum* phycobilisomes showed optimum long term stability, as determined from the preservation of energy transfer, between pH 7.5 and 9.0, at ionic strengths equivalent to or higher than that of 0.75 M Na-K-PO_4, pH 8.0. These phycobilisomes were much less stable at pH 7.0. A procedure based on these observations was developed for the preparation of *Synechococcus* sp. phycobilisomes. This procedure leads to little if any dissociation of phycobilisomes and results in particles with a fluorescence emission maximum at 673 nm.

Cell breakage and the initial differential centrifugation (see "Methods") are carried out in 0.65 M Na-K-PO_4, pH 8.0. Use of buffers of higher salt concentration resulted in a loss of large amounts of phycobiliproteins in the pellet. The sucrose step gradients were 0.75 M in Na-K-PO_4, pH 8.0, and the phycobilisomes isolated from these gradients were stable for several weeks when stored at 4°C. The choice of the proportion of the counter ions, Na^+ and K^+, was dictated primarily by the solubility of the mono- and dibasic salts at 0.75 M and pH 8.0.

In an attempt to avoid the high buffering and chelating properties of Na-K-PO_4, 0.8 M sodium sulfate in 0.05 M Tris-Cl at pH 8.0 was examined. This mixture permitted isolation of phycobilisomes equivalent in stability to those prepared in the phosphate buffer, as judged by fluorimetry and electron microscopy. The crystallization of sodium sulfate at low temperatures was a disadvantage in the use of this salt.

Phycobilisome isolations were carried out both at 4° and at 18°C. The clusters appeared to be slightly better preserved in the preparations obtained at 18°C, but the difference was minor. The major advantage of the 18°C procedure was the removal of a large amount of chlorophyll-containing material in the 31,000 × *g* differential centrifugation step. As shown in Fig. 1, the 680 nm absorbance decreased by about 90% after centrifugation at 18°C. At the higher temperature, a membrane/detergent fraction was observed which floated on top of the aqueous solution containing the phycobilisomes. At 4°C, the chlorophyll-containing material was completely dispersed in the 0.65 M Na-K-PO_4, pH 8.0. A major advantage of the partitioning prior to the sucrose gradient centrifugation was that a higher load of cell extract could be applied to the gradient without problems due to membrane aggregates spreading through the gradient during centrifugation.

The conditions of optimal stability for *Synechococcus* sp. 6301 phycobilisomes (pH 7.5 to 9.0, and a very high ionic strength) are consistent with a number of observations made by other investigators on the physiology of this organism. Masamoto and Nishimura (28) and Falkner *et al.* (29) estimated the cytoplasmic pH of cells incubated in the light to be 8.5 and 7.5, respectively. Utkilen *et al.* (30) and Jeanjean and Broda (31) found that the optimum pH for sulfate uptake by whole cells lay in the range of 8.0 to 8.5. An interesting study by Herzfeld and Zillig (32) on the *in vitro* reassembly of dissociated RNA polymerase subunits from this organism revealed an optimum pH of 8.0 to 8.5 and a high ionic strength requirement. At least 0.4 M KCl was required for reassembly as contrasted to 0.15 to 0.3 M KCl for the reactivation of the *E. coli* enzyme.

Tandeau de Marsac and Cohen-Bazire (8) demonstrated the presence of several colorless polypeptides in cyanobacterial phycobilisomes. These components were grouped into three molecular weight categories: M_r = 70 to 120,000 (I), 30 to 70,000 (II), and 25 to 30,000 (III). When cells were broken in low ionic strength buffers and the phycobilisomes were dissociated, Group I polypeptides were shown to partition with the membrane fraction on differential centrifugation, whereas polypeptides of Groups II and III, as well as the phycobiliproteins were all present in the soluble fraction (8). Further evidence that colorless polypeptides were integral components of phycobilisomes was provided principally by studies of the effect of "chromatic adaptation" on the structure of these particles. In certain cyanobacteria, the synthesis of phycoerythrin and phycocyanin is regulated by the wavelength of the incident radiation (33, 34). In green light, such organisms suppress the synthesis of phycocyanin, whereas in red light, phycoerythrin is not made. Tandeau de Marsac and Cohen-Bazire (8) described the striking finding that in phycobilisomes isolated from cells grown in red light, drastic decrease in phycoerythrin content was paralleled by decrease in certain of the Group II colorless polypeptides. Likewise, in phycobilisomes from cells grown in green light, the decrease in phycocyanin content was associated with a decrease in the others of the Group II colorless polypeptides.

The above findings were challenged by Gantt (2) and by Mörschel *et al.* (5), who failed to detect colorless polypeptides in the phycobilisomes of the red algae *P. cruentum* and *Rhodella violacea* (35) and in those of the filamentous cyanobacterium *Nostoc* sp. (36).

Our experiments, discussed in detail under "Results," establish that colorless polypeptides are indeed components of *Synechococcus* sp. 6301 phycobilisomes. We have also examined the phycobilisomes of three other cyanobacteria, *Anabaena* sp. 6411, *Aphanocapsa* sp. 6701, and *Synechococcus* sp. 6312, as well as of two unicellular red algae *P. cruentum* and *P. aerugineum*. In each instance, the presence of colorless polypeptides could be readily demonstrated.[2] The failure of Gantt and co-workers (7, 36), and of Koller *et al.* (35) to detect these components appears to be due to the nature and low resolving power of the polyacrylamide gel electrophoresis systems that these workers had chosen to examine the composition of dissociated phycobilisomes.

[2] J. Gingrich, G. Yamanaka, and A. N. Glazer, unpublished observations.

As shown in Fig. 5*A*, the phycobilisomes of *Synechococcus* sp. 6301 are clusters of rods which frequently come together at right angles. Side-by-side association of the rods was rarely seen. These phycobilisomes have a much more open structure than the compact phycobilisome particles derived from the phycoerythrin-containing organisms, *P. cruentum* (4), *R. violacea* (5), and *Nostoc* (36). Our study provides clear evidence of association between the presence of the cluster structure and energy transfer, as illustrated by the dissociation study at pH 5.5 (presented in Fig. 5, *B* and *C* and Fig. 6, *inset B*).

The information on the spatial arrangement of the majority of the components of the phycobilisome has yet to be obtained. The rods bear a striking resemblance to short stacks of phycocyanin previously seen in crude cell extracts of *Synechococcus* sp. 6301 (26). Since phycocyanin represents 75% of the protein in the phycobilisome, it is plausible to assume that the rods are made up of stacks of disc-shaped phycocyanin hexamers (1 to 5 hexamers high). They may contain other components as well.

The spectroscopic data on intact phycobilisomes supports the view that phycocyanin is present in the $(\alpha\beta)_6$ form in these particles. The similarity between the circular dichroism spectrum of intact phycobilisomes and of hexameric phycocyanin (15) is very close. Phycocyanin accounts for about 90% of the absorbance of phycobilisomes in the region of interest. It is noteworthy that dissociation of phycobilisomes, under conditions where phycocyanin hexamers are stable, does not affect the circular dichroism or the absorption spectrum of the sample. We can conclude that no new strong interactions involving phycocyanin bilin chromophores arise as a consequence of assembly into phycobilisomes.

Limited treatment of intact phycobilisomes with trypsin leads to rapid cleavage of polypeptides 1 and 2 (Fig. 3), with little change in the ultrastructure or fluorescence emission properties of these particles. As discussed above, Tandeau de Marsac and Cohen-Bazire (8) concluded that polypeptides in this molecular weight range were involved in the attachment of phycobilisomes to the photosynthetic lamellae. The exposed location of these polypeptides indicated by their high susceptibility to tryptic degradation is consistent with this view.

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